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Synthesis and Biological Activities of Novel Antiallergic Agents with 5-Lipoxygenase Inhibiting Action

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Abstract—Novel benzimidazole derivatives were synthesized and their pharmacological activities were examined. These compounds showed a good suppressive action on histamine release from rat peritoneal mast cells produced by antigen–antibody reaction, an antagonistic action on guinea pig ileum contraction caused by histamine, an inhibitory action on 5-lipoxygenase in rat basophilic leukemia-1 (RBL-1) cells, and a preventive action on NADPH dependent lipid peroxidation induced by Fe^{3+}-ADP in rat liver microsomes. In addition, 1-[2-[2-(4-Hydroxy-2,3,5-trimethylphenoxy)ethoxy]-ethyl]-2-(4-methyl-1-homopiperazino)-1*H*-benzimidazole difumarate (BOM1006) exhibited a dose dependent suppressive action on 48 h homologous passive cutaneous anaphylaxis (PCA) reaction in rats orally administered the drug.

Introduction

In recent years, a rapid increase in the number of patients with some allergic symptoms has become our grave concern. Degranulation of mast cell induced by antigen–antibody reaction triggers allergic disease of type I including bronchial asthma, allergic rhinitis, atopic dermatitis, and pollenosis. Histamine is one of the chemical mediators released in this reaction and combines with H_1 receptors of tissues to cause smooth muscle contraction, vasodilatation, and vascular permeability rising, thereby leading to allergic symptoms.[1] The action mechanism for most of the conventional antiallergic agents was to suppress the release of chemical mediators, mainly histamine, and to antagonize them. However, these actions alone were unable to prevent the diseases from worsening.

Leukotrienes are chemical mediators deeply involved in allergic inflammation and have activities to accumulate inflammatory cells and contract bronchial smooth muscles.[2,3] Some drugs developed as peptide leukotrienes receptor antagonists have been clinically used successfully.[4,5] Involvement of 5-lipoxygenase is known in the biosynthesis of leukotrienes, and some agents that inhibit the synthesis have been developed.[6,7] Active oxygen is released from inflammatory cells such as eosinophils in allergic inflammation and this has been regarded as a cause of worsening symptoms.[8–10]

We previously synthesized trimethylhydroquinone derivatives with an antihistaminic action, an inhibitory action on 5-lipoxygenase, and an antioxidative action and reported their effectiveness in asthma model animals.[11,12] We then synthesized novel antiallergic agents with multiple pharmacological activities through hybridization of emedastine, known as an antiallergic agent that possesses a suppressive action on histamine release from mast cells and an antagonistic action on H_1 receptor[13–15] as the core, benzimidazole derivatives, and trimethylhydroquinone derivatives that have an inhibitory action on 5-lipoxygenase and an antioxidative action.[16] In this article, we report synthesis and the pharmacological activities of the three novel benzimidazole derivatives which were considered most promising in the previous paper. The activities studied are suppressive action on histamine release from rat peritoneal mast cells caused by antigen–antibody reaction, antagonistic action on contraction of guinea pig ileum produced by

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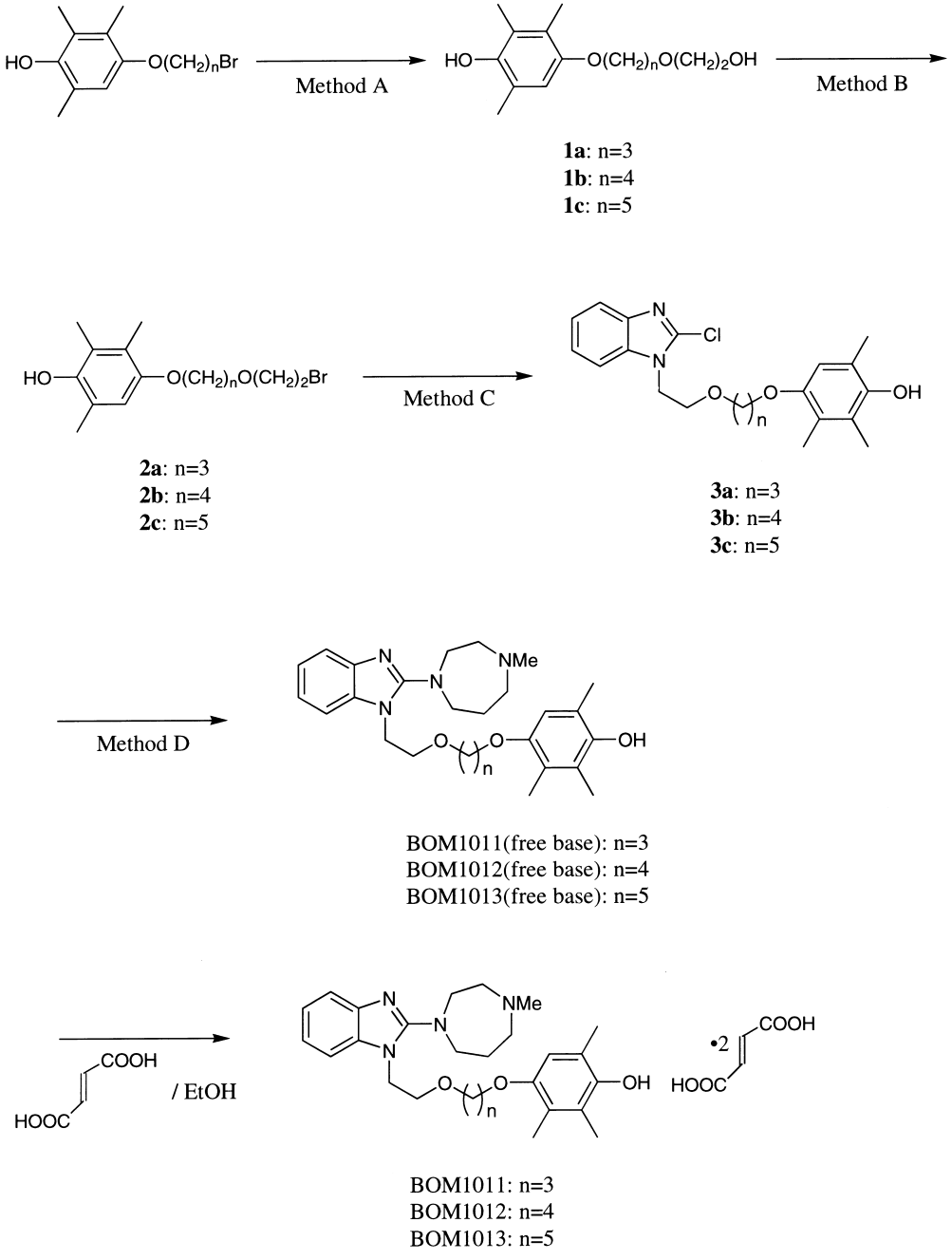
histamine, inhibitory action on 5-lipoxygenase in rat basophilic leukemia-1 (RBL-1) cells, and preventive action on NADPH dependent lipid peroxidation induced by Fe^{3+}-ADP in rat liver microsomes. Moreover, descriptions are given on the suppressive action of 1-[2-[2-(4-hydroxy-2,3,5-trimethylphenoxy)ethoxy]ethyl]-2-(4-methyl-1-homopiperazino)-1*H*-benzimidazole difumarate (BOM1006) on rat 48 h homologous passive cutaneous anaphylaxis (PCA) reaction.

Chemistry

It was shown in the previous paper, that when trimethylhydroquinone derivatives are hybridized with benzimidazole derivatives so that the resultant hybrids bear several antiallergic pharmacological activities, the site of hybridization is restricted and only BOM1006 exhibits an antihistaminic activity which has two ether groups in the spacer between benzimidazole ring and trimethylhydroquinone, thus suggesting that this is a most promising hybrid form.[16] Hence, we newly designed benzimidazole derivatives (BOM1011–1013) with different alkyl chain lengths between ether groups in the spacer. The synthetic route is shown in Scheme 1. Compounds **1a**–**1c** were obtained by stirring a mixture of ethyleneglycol and 4-(bromoalkyloxy)-2,3,6-trimethylphenol in the presence of NaOH at 45–60 °C for 3–6 h (Method A). The products were brominated with CBr_4 and Ph_3P to give compounds **2a**–**2c** (Method B),



Scheme 1.

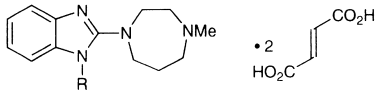
which were then introduced into 2-chloro-1*H*-benzimidazole according to the method described in the previous paper, to yield compounds **3a**–**3c** (Method C). BOM1011–1013 free bases were obtained through the condensation reaction of **3a**–**3c** with 1-methylhomopiperazine (Method D). Finally, the products were converted to their difumarates (BOM1011–1013), which were then used in pharmacological examinations.

Results and Discussion

The suppressive action of the benzimidazole derivatives (BOM1006, 1011–1013) was examined on histamine release from rat peritoneal mast cells induced by antigen–antibody reaction. Mast cells were withdrawn from rat peritoneal cavity passively sensitized with rat anti-2,4-dinitrophenyl-bovine serum albumin (DNP-BSA) serum and the cells were incubated with each of the test compounds at 37 °C, 15 min. Degranulation of the cells was then induced through their antigen–antibody reaction with DNP-BSA added as the antigen during 20 min incubation at 37 °C. The amounts of histamine in mast cells and released from the cells were determined fluorophotometrically using *o*-phthalaldehyde as the reagent, and the rate of suppression for the test compounds was calculated against the control on the basis of the release rate data of histamine thus obtained. All of the benzimidazole derivatives synthesized and emedastine showed histamine release suppressing action at their concentration of 10^{-5} M (Fig. 1). The suppressive action of these benzimidazole derivatives was as strong as that of emedastine. This suggests little or no effect of the introduction of trimethylhydroquinone moiety and its spacer length on the histamine release suppressing action of benzimidazole derivatives.

Next, the H_1 receptor antagonizing action of the benzimidazole derivatives synthesized was examined through contraction of guinea pig ileum by histamine. Guinea pig ileum was incubated with each of the test compounds for 5 min and then given histamine in an accumulative way. The values of pA_2 obtained were shown in Table 1. The benzimidazole derivatives synthesized, exhibited a good H_1 receptor antagonizing action, though the action was somewhat weaker than that of emedastine. Little or no effect of the length of spacer was also shown on this action.

Table 1. Effect of benzimidazole derivatives and emedastine on histamine induced contraction of isolated ileum from guinea pig



Compd.	R	pA_2[a]
Emedastine	—$(CH_2)_2OCH_2CH_3$	9.09
BOM1006	—$(CH_2)_2O(CH_2)_2O$—C$_6$H$_2$(CH$_3$)$_3$—OH	7.49
BOM1011	—$(CH_2)_2O(CH_2)_3O$—C$_6$H$_2$(CH$_3$)$_3$—OH	7.17
BOM1012	—$(CH_2)_2O(CH_2)_4O$—C$_6$H$_2$(CH$_3$)$_3$—OH	6.87
BOM1013	—$(CH_2)_2O(CH_2)_5O$—C$_6$H$_2$(CH$_3$)$_3$—OH	7.11

[a]Test compound concentration, 10^{-6}–10^{-9} M. Results are the means of triplicate determinations.

The inhibitory action of the synthesized derivatives was then examined on 5-lipoxygenase in RBL-1 cells. Arachidonic acid was added to a homogenate of the cells and the amount of 5-hydroxy-6,8,11,14-eicosatetraenoic

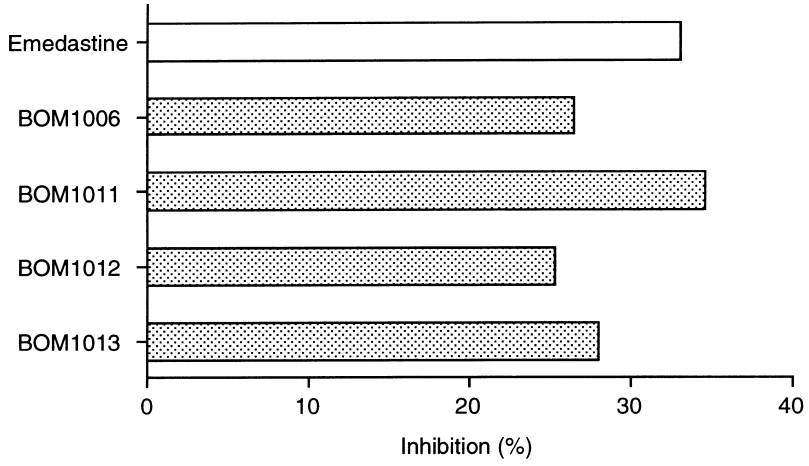


Figure 1. Effect of benzimidazole derivatives and emedastine on histamine release from sensitized rat peritoneal mast cell caused by antigen. Test compound concentration, 10^{-5} M. Results are the means of duplicate determinations.

acid (5-HETE) produced was determined by HPLC. The rate of inhibition for the test compounds against the control was calculated using the analytical data obtained. As a result, all of the benzimidazole derivatives synthesized were found to show a concentration dependent inhibitory action on 5-lipoxygenase at their concentration 3×10^{-6} M–10^{-7} M and the value of IC_{50} ranged from 4.38×10^{-7} to 9.48×10^{-7} M (Fig. 2). Meanwhile, their inhibitory action was slightly weaker than that of nordihydroguaiaretic acid (NDGA) as a positive control at 3×10^{-7} M. Hence, even though the introduction of trimethylhydroquinone moiety additionally gave an inhibitory action on 5-lipoxygenase to benzimidazole derivatives, this action was also suggested to be hardly affected by the spacer length.

An examination was performed of the preventive action of the synthesized derivatives on NADPH dependent lipid peroxidation induced by Fe^{3+}-ADP in rat liver microsomes as an index of antioxidative action. The amount of peroxidized lipids produced in rat liver microsomes was determined as the amount of malondialdehyde using 2-thiobarbituric acid (TBA) as the reagent, and the rate of inhibition against the control was calculated on the basis of the analytical data obtained. All synthesized benzimidazole derivatives exhibited a concentration dependent preventive action on lipid peroxidation at their concentration 5×10^{-6} M–10^{-6} M and the value of IC_{50} varied from 1.87×10^{-6} to 2.58×10^{-6} M (Fig. 3). Moreover, these synthesized derivatives had a preventive action stronger than that of butylated hydroxytoluene (BHT) as a positive control at 3×10^{-6} M. Thus, the introduction of trimethylhydroquinone moiety was shown to give an antioxidative activity to benzimidazole derivatives, in addition to 5-lipoxygenase inhibiting activity. The spacer length

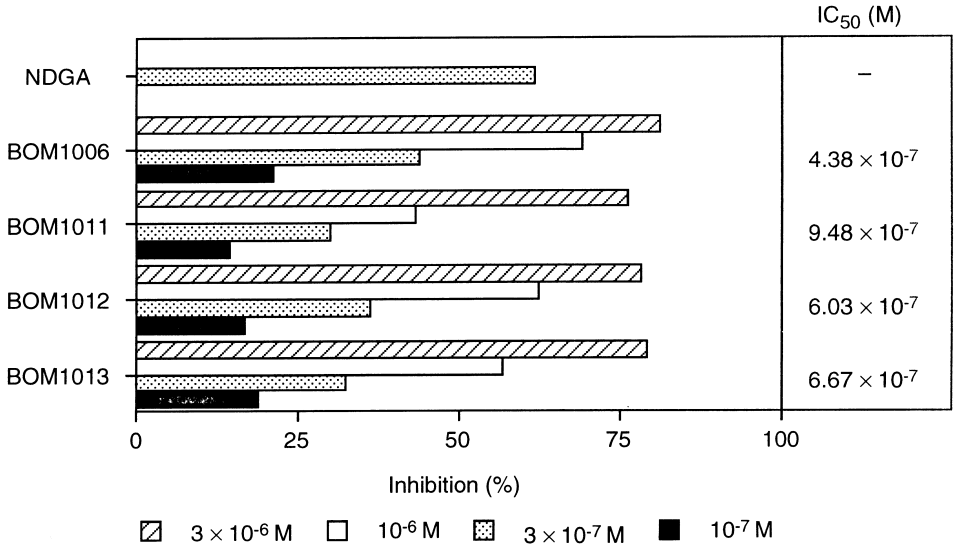


Figure 2. Effect of benzimidazole derivatives and NDGA on RBL-1 cell 5-lipoxygenase activity. Results are the means of duplicate determinations.

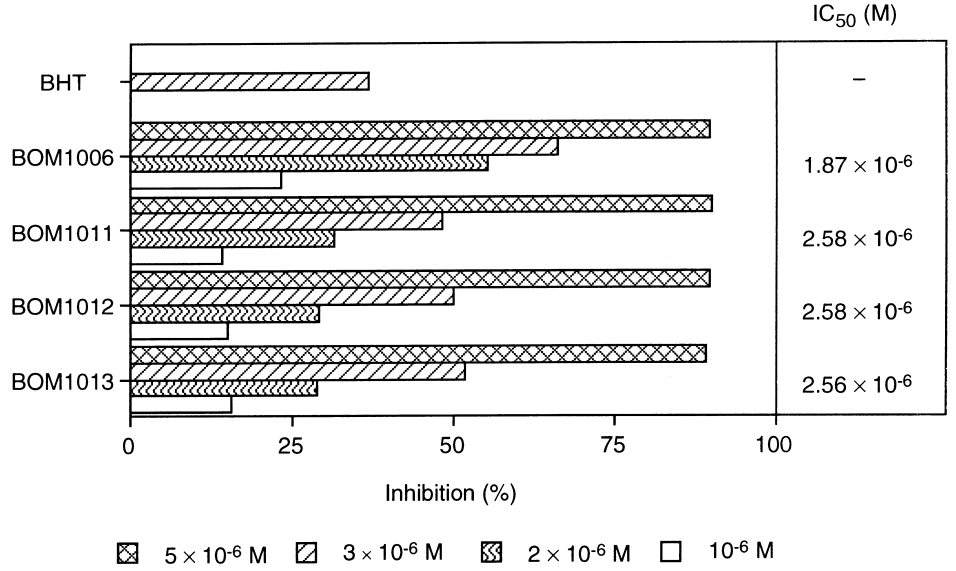


Figure 3. Effect of benzimidazole derivatives and BHT on Fe^{3+}-ADP induced NADPH dependent lipid peroxidation in rat liver microsomes. Results are the means of duplicate determinations.

was again suggested to have almost no effect on this activity.

Finally, an examination was conducted to check if BOM1006 has an in vivo antiallergic activity through its action on 48 h homologous PCA reaction in rat dorsal skin. Rats were subcutaneously administered rat anti-DNP-BSA serum in their back and 48 h later intravenously administered saline solution containing Evans blue and DNP-BSA. The animals were sacrified half an hour later by bloodletting, the part of their dorsal skin that received the antibody was withdrawn, and the rate of suppression for the agent was calculated against the control, using the spectrophotometrically determined concentrations of the dye in the solutions obtained by extracting it from the withdrawn skin part.

Each of the test compounds was orally given to the animals an hour before the antigen administration. BOM1006 was found to suppress PCA reaction in a dose dependent way at doses from 3 to 30 mg/kg (Fig. 4). The compound thus manifested itself to be a good antiallergic agent in vivo even though its action was slightly weaker than that of emedastine.

In conclusion, we have demonstrated that the novel benzimidazole derivatives possess multiple pharmacological activities and BOM1006 is effective in allergic model animals when it is orally administered. BOM1006 may be a leading compound in the future development of new antiallergic agents.

Experimental

Chemistry

^{1}H NMR spectra were measured on a JEOL JNM-EX270 (270 MHz) spectrometer in $CDCl_3$ solution with tetramethylsilane as an internal standard. FABMS were obtained on a JEOL JMS-AX505H spectrometer.

BOM1006 has been described previously.[16]

General method A. 4-[3-(2-Hydroxyethoxy)propyloxy]-2,3,6-trimethylphenol (1a). 4-(3-Bromopropyloxy)-2,3,6-trimethylphenol[11] (5.9 g, 21 mmol), ethyleneglycol (13.4 g, 216 mmol) and NaOH (867 mg, 21 mmol) were mixed and stirred at 45 °C for 3 h. The mixture was added to water, extracted with $CHCl_3$, washed with water and brine, dried over anhydrous Na_2SO_4 and evaporated to afford **1a** (4.3 g, 16.9 mmol, 80%) as a brown oil, ^{1}H NMR δ: 1.94 (1H, t, J = 5.61 Hz), 2.00–2.08 (2H, m), 2.13 (3H, s), 2.15 (3H, s), 2.22 (3H, s), 3.82–3.86 (2H, m), 3.91 (2H, q, J = 5.61 Hz), 4.01–4.06 (4H, m), 4.34 (1H, s), 6.34 (1H, s). FABMS m/z: 255 ($M^+ + 1$).

Compounds **1b** and **1c** were prepared in the same manner as **1a** (General method A).

4-[4-(2-Hydroxyethoxy)butoxy]-2,3,6-trimethylphenol (1b). 4-(4-Bromobutoxy)-2,3,6-trimethylphenol (8.0 g, 28 mmol), ethyleneglycol (17.3 g, 279 mmol) and NaOH (1.1 g, 28 mmol) were mixed and stirred at 45 °C for 5 h. **1b** (3.9 g, 14.6 mmol, 52%); pale brown oil, ^{1}H NMR δ: 1.74–1.90 (4H, m), 1.93–2.00 (2H, m), 2.14 (3H, s), 2.17 (3H, s), 2.22 (3H, s), 3.73 (2H, t, J = 6.27 Hz), 3.89 (2H, t, J = 5.61 Hz), 4.02–4.06 (2H, m), 4.36 (1H, s), 6.34 (1H, s). FABMS m/z: 269 ($M^+ + 1$).

4-[5-(2-Hydroxyethoxy)pentyloxy]-2,3,6-trimethylphenol (1c). 4-(5-Bromopentyloxy)-2,3,6-trimethylphenol (10.0 g, 33 mmol), ethyleneglycol (21.0 g, 338 mmol) and NaOH (1.4 g, 35 mmol) were mixed and stirred at 60 °C for 6 h. **1c** (5.6 g, 19.9 mmol, 60%); brown oil, ^{1}H NMR δ: 1.52–1.68 (5H, m), 1.73–1.85 (2H, m), 1.92–1.98 (2H, m), 2.14 (3H, s), 2.16 (3H, s), 2.22 (3H, s), 3.69 (2H, t, J = 6.27 Hz), 3.88 (2H, t, J = 6.27 Hz), 4.00–4.04 (2H, m), 4.34 (1H, s), 6.34 (1H, s). FABMS m/z: 283 ($M^+ + 1$).

General method B. 4-[3-(2-Bromoethoxy)propyloxy]-2,3,6-trimethylphenol (2a). 1a (4.0 g, 15 mmol) and CBr_4 (6.5 g, 19 mmol) were dissolved in 30 mL of $CHCl_3$. Ph_3P (5.0 g, 19 mmol) was added in the mixture on ice bath. The mixture was stirred at room temperature for 40 min and evaporated. The residue was

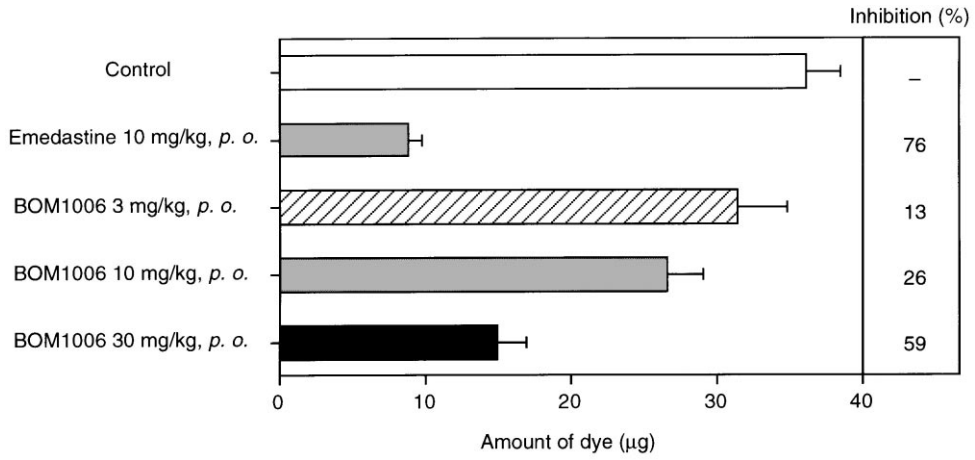


Figure 4. Effect of BOM1006 and emedastine on 48 h homologous PCA reaction in rats. Each value represents the mean and S. E. of 3 to 5 rats.

chromatographed on silica gel (hexane:AcOEt, 3:1) to afford **2a** (3.3 g, 10.4 mmol, 69%) as a brown oil, ^{1}H NMR δ: 2.13 (3H, s), 2.17 (3H, s), 2.22 (3H, s), 2.26–2.36 (2H, m), 3.63 (2H, t, J = 6.58 Hz), 3.80–3.84 (2H, m), 4.01–4.05 (4H, m), 4.30 (1H, s), 6.80 (1H, s). FABMS m/z: 318 ($M^+ + 2$).

Compounds **2b** and **2c** were prepared in the same manner as **2a** (General method B).

4-[4-(2-Bromoethoxy)butoxy]-2,3,6-trimethylphenol (2b). Compounds **1b** (4.4 g, 16 mmol) and CBr_4 (6.5 g, 19 mmol) were dissolved in 30 mL of $CHCl_3$. Ph_3P (5.0 g, 19 mmol) was added in the mixture on ice bath. The mixture was stirred at room temperature for 40 min. **2b** (3.8 g, 11.5 mmol, 72%); brown oil, ^{1}H NMR δ: 1.90–1.97 (2H, m), 2.03–2.13 (2H, m), 2.12 (3H, s), 2.17 (3H, s), 2.21 (3H, s), 3.49 (2H, t, J = 6.60 Hz), 3.82–3.86 (2H, m), 3.91 (2H, t, J = 5.94 Hz), 4.03–4.06 (2H, m), 4.30 (1H, s), 6.80 (1H, s). FABMS m/z: 333 ($M^+ + 2$).

4-[5-(2-Bromoethoxy)pentyloxy]-2,3,6-trimethylphenol (2c). 1c (4.9 g, 17 mmol) and CBr_4 (7.0 g, 21 mmol) were dissolved in 30 mL of $CHCl_3$. Ph_3P (5.5 g, 21 mmol) was added in the mixture on an ice bath. The mixture was stirred at room temperature for 1 h. **2c** (3.7 g, 10.7 mmol, 63%); brown oil, ^{1}H NMR δ: 1.62–1.68 (2H, m), 1.75–1.86 (2H, m), 1.88–1.99 (2H, m), 2.10 (3H, s), 2.14 (3H, s), 2.22 (3H, s), 3.45 (2H, t, J = 6.93 Hz), 3.81–3.86 (4H, m), 4.00–4.06 (2H, m), 4.26 (1H, s), 6.51 (1H, s). FABMS m/z: 347 ($M^+ + 2$).

General method C. 2-Chloro-1-[2-[3-(4-hydroxy-2,3,5-trimethylphenoxy)-propyloxy]ethyl]-1*H*-benzimidazole (3a). 2-Chloro-1*H*-benzimidazole (2.0 g, 13 mmol) was dissolved in 20 mL of *N*,*N*-dimethylformamide (DMF). 60%NaH dispersion in mineral oil (600 mg, 13 mmol) was added to the solution on ice bath and stirred for 30 min. To the mixture was added **2a** (4.2 g, 13 mmol) and stirred at 80 °C for 4 h. The mixture was added to water, extracted with AcOEt, washed with water and brine, dried over anhydrous Na_2SO_4 and evaporated. The residue was washed with hexane:AcOEt (1:1) to afford **3a** (2.4 g, 6.2 mmol, 48%) as a pale brown amorphous powder, ^{1}H NMR δ: 2.08 (3H, s), 2.16 (3H, s), 2.18–2.24 (2H, m), 2.21 (3H, s), 3.70–3.75 (2H, m), 3.82–3.98 (4H, m), 4.32 (2H, t, J = 5.62 Hz), 4.46 (1H, s), 6.48 (1H, s), 7.23–7.27 (2H, m), 7.37–7.41 (1H, m), 7.66–7.69 (1H, m). FABMS m/z: 389 ($M^+ + 1$).

Compounds **3b** and **3c** were prepared in the same manner as **3a** (General method C).

2-Chloro-1-[2-[4-(4-hydroxy-2,3,5-trimethylphenoxy)butoxy]ethyl]-1*H*-benzimidazole (3b). 2-Chloro-1*H*-benzimidazole (2.0 g, 13 mmol) was dissolved in 20 mL of DMF. 60% NaH dispersion in mineral oil (600 mg, 13 mmol) was added to the solution on ice bath and stirred for 30 min. The mixture was added **2b** (4.2 g, 13 mmol) and stirred at 80 °C for 4 h. **3b** (1.9 g, 4.7 mmol, 36%); pale brown amorphous powder, ^{1}H NMR δ: 1.82–1.88 (2H, m), 1.97–2.10 (2H, m), 2.10 (3H, s), 2.14 (3H, s), 2.22 (3H, s), 3.69–3.74 (2H, m), 3.82–3.98 (4H, m), 4.34 (2H, t, J = 5.62 Hz), 4.46 (1H, s), 6.46 (1H, s), 7.24–7.28 (2H, m), 7.36–7.40 (1H, m), 7.66–7.68 (1H, m). FABMS m/z: 403 ($M^+ + 1$).

2-Chloro-1-[2-[5-(4-hydroxy-2,3,5-trimethylphenoxy)pentyloxy]ethyl]-1*H*-benzimidazole (3c). 2-Chloro-1*H*-benzimidazole (2.0 g, 13 mmol) was dissolved in 20 mL of DMF. 60% NaH dispersion in mineral oil (600 mg, 13 mmol) was added to the solution on ice bath and stirred for 30 min. The mixture was added **2c** (4.5 g, 13 mmol) and stirred at 80 °C for 4 h. **3c** (1.4 g, 3.4 mmol, 26%); pale brown amorphous powder, ^{1}H NMR δ: 1.56–1.76 (2H, m), 1.78–1.97 (4H, m), 2.08 (3H, s), 2.14 (3H, s), 2.21 (3H, s), 3.64–3.74 (2H, m), 3.82–3.96 (4H, m), 4.32 (2H, t, J = 5.61 Hz), 4.44 (1H, s), 6.46 (1H, s), 7.22–7.26 (2H, m), 7.36–7.41 (1H, m), 7.64–7.66 (1H, m). FABMS m/z: 417 ($M^+ + 1$).

General method D. 1-[2-[3-(1-Hydroxy-2,3,6-trimethylphenoxy)propyloxy]ethyl]-2-(4-methyl-1-homopiperazino)-1*H*-benzimidazole (BOM1011 (free base)). Compound **3a** (1.2 g, 3.1 mmol) and 1-methylhomopiperazine (927 mg, 9.2 mmol) were mixed at 130 °C for 3 h. The mixture was chromatographed on silica gel ($CHCl_3$:MeOH, 50:1) to afford BOM1011 (1.2 g, 2.6 mmol, 84%) as a brown oil, ^{1}H NMR δ: 1.74–1.92 (2H, m), 2.00–2.14 (2H, m), 2.10 (3H, s), 2.14 (3H, s), 2.20 (3H, s), 2.38 (3H, s), 2.70–2.88 (4H, m), 3.62–3.68 (4H, m), 3.74–3.79 (2H, m), 3.82–3.97 (4H, m), 4.23 (2H, t, J = 6.28 Hz), 6.44 (1H, s), 7.08–7.17 (2H, m), 7.23–7.28 (1H, m), 7.54 (1H, dd, J = 1.21, 6.95 Hz). FABMS m/z: 467 ($M^+ + 1$).

BOM1012 and BOM1013 were prepared in the same manner as BOM1011 (General method D).

1-[2-[4-(1-Hydroxy-2,3,6-trimethylphenoxy)butoxy]ethyl]-2-(4-methyl-1-homopiperazino)-1*H*-benzimidazole (BOM 1012 (free base)). Compound **3b** (1.2 g, 2.9 mmol) and 1-methylhomopiperazine (880 mg, 8.8 mmol) were mixed at 130 °C for 3 h. BOM1012 (1.0 g, 2.1 mmol, 72%); brown oil, ^{1}H NMR δ: 1.36–1.42 (2H, m), 1.64–1.83 (2H, m), 2.00–2.04 (2H, m), 2.11 (3H, s), 2.14 (3H, s), 2.21 (3H, s), 2.40 (3H, s), 2.68–2.82 (4H, m), 3.62–3.67 (4H, m), 3.78–3.90 (2H, m), 3.92–3.98 (4H, m), 4.26 (2H, t, J = 6.25 Hz), 6.42 (1H, s), 7.06–7.14 (2H, m), 7.22–7.26 (1H, m), 7.52 (1H, dd, J = 1.32, 6.72 Hz). FABMS m/z: 481 ($M^+ + 1$).

1-[2-[5-(1-Hydroxy-2,3,6-trimethylphenoxy)pentyloxy]-ethyl]-2-(4-methyl-1-homopiperazino)-1*H*-benzimidazole (BOM1013 (free base)). Compound **3c** (1.2 g, 2.8 mmol) and 1-methylhomopiperazine (860 mg, 8.6 mmol) were mixed at 130 °C for 5 h. BOM1013 (1.1 g, 2.2 mmol, 79%); brown oil, ^{1}H NMR δ: 1.41–1.58 (2H, m), 1.72–1.86 (4H, m), 2.01–2.11 (2H, m), 2.13 (3H, s), 2.16 (3H, s), 2.22 (3H, s), 2.41 (3H, s), 2.68–2.84 (4H, m), 3.63–3.66 (4H, m), 3.76–3.90 (2H, m), 3.94–3.98 (4H, m), 4.24 (2H, t, J = 6.24 Hz), 6.44 (1H, s), 7.04–7.13 (2H, m), 7.20–7.24 (1H, m), 7.51 (1H, dd, J = 1.28, 6.68 Hz). FABMS m/z: 495 ($M^+ + 1$).

Synthesis of benzimidazole derivative difumarate (BOM 1011–1013). Free base of benzimidazole derivative

was dissolved in EtOH. Fumaric acid (2.2 eq) in hot EtOH solution was added. The mixture was cooled, evaporated, washed with hexane and dried to afford the benzimidazole derivative difumarate (BOM1011–1013).

Pharmacology

Materials. Male Wistar rats and male Hartley guinea pigs were obtained from Japan SLC Inc. DNP-BSA and rat anti-DNP-BSA serum were prepared according to the method described in a previous paper.[17] RBL-1 cell was obtained from Dainippon Pharmaceutical Co., Ltd. 5-HETE and L-α-phosphatidyl-L-serine were obtained from Sigma Chemical Co. ADP, histamine dihydrochloride and β-NADPH were obtained from Wako Pure Chemical Industries, Ltd. Arachidonic acid, BHT and NDGA were obtained from Nacalai Tesque, Inc.

Assay of histamine release from sensitized rat peritoneal exudate cell caused by antigen.[17] Male Wistar rats weighing about 300 g were passively sensitized by an intraperitoneal injection of 2 mL of physiological saline containing 10 μL of rat anti-DNP-BSA serum (titer; 1:2000 or greater). After 2 days, rats were exsanguinated and injected intraperitoneally with 15 mL of Tyrode solution containing 10 mM HEPES and 0.05% gelatin (pH 7.4). The abdominal region was gently massaged for 1.5 min. The peritoneal exudate cells were collected and recovered by washing the cavity with 15 mL of Tyrode solution. Cells were washed 3 times with Tyrode solution by centrifugation ($55\times g$) at 4 °C for 8 min and resuspended in a small volume of Tyrode solution. Mast cells were counted after staining with toluidin blue.

A Tyrode solution containing 10 mM HEPES and 0.05% gelatin (pH 7.4) was added 30 μg/mL L-α-phosphatidyl-L-serine and test compound, and preincubated at 37 °C for 5 min. After adding 10^5 mast cells/mL peritoneal exudate cells to the preincubated solution, the resultant solution was incubated at 37 °C for 15 min. To the incubated solution was added 10 μg protein/ml DNP-BSA and the solution was further incubated at 37 °C for 20 min (final volume 1 mL). After ice-cooling for 10 min, the solution was centrifuged at 4 °C for 10 min ($300 \times g$). An aliquot (0.5 mL) of the supernatant liquid was used to determine the amount of released histamine after adding 0.5 mL of 0.2 N HCl. To the precipitate was added 2 mL of 0.1 N HCl and the resultant dispersion was treated at 100 °C for 10 min. After ice-cooling for 10 min, the dispersion was centrifuged at 4 °C for 10 min ($780 \times g$), an aliquot (1 mL) of the supernatant liquid of which was then used to determine the amount of histamine associated with the cells. Determinations of the amount of histamine were carried out according to the method of Shore et al.[18] Thus, 0.1 mL of 2 N NaOH and 0.05 mL of 1% *o*-phthalaldehyde MeOH solution were added to 1 mL of test sample, which was then allowed to stand at room temperature for 4 min. After adding 0.1 mL of 2 M citrate, the fluorescence intensity of the sample was measured (EX 356 nm, EM 440 nm) and the rate of histamine release was calculated.

Measurement of histamine induced contraction of isolated ileum from guinea pig. Male Hartley guinea pigs weighing 300–400 g were sacrificed and the ilea were prepared. The segments (about 1 cm) of ileum were suspended in a Magnus tube (35 °C, 95% O_2/5% CO_2) containing 10 mL of Tyrode solution. After 30 min, test compound was added and incubated for 5 min. Histamine was cumulatively added to the tube to obtain a concentration-response curve. The contractile responses were measured using an isotonic transducer (TD-112S; Nihon Kohden Co., Ltd.). pA_2 values of test compounds were calculated by the method of Takayanagi.[19]

Measurement of RBL-1 cell 5-lipoxygenase activity.[20] The modified method of Blackham et al.[21] was used. RBL-1 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated newborn calf serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin. Cells were cultured at 37 °C in 5% CO_2/air. Cells in the growth phase (5×10^5–10^6 cells/mL) were collected by centrifugation ($100 \times g$, 5 min) and suspended at 3×10^7 cells/mL in 50 mM phosphate buffer (0.25 M sucrose, 1 mM EDTA, 2 mM glutathione, pH 7.4). Cells were stored at −80 °C until use. The assay system (0.5 mL) consisted of 50 mM phosphate buffer (0.25 M sucrose, 1 mM EDTA, 2 mM glutathione, pH 7.4), the test compound in 1% dimethyl sulfoxide (DMSO), 2 mM $CaCl_2$, 0.2 mg/mL arachidonic acid (10 mg/mL MeOH soln; 10 μL) and 10^7 cells/mL RBL-1 cells homogenate. Reaction mixture was incubated at 37 °C for 3 min, and then 0.5 mL of MeOH was added to terminate the reaction. The mixture was centrifuged ($2000 \times g$, 15 min), 5-HETE in the supernatant was analyzed by HPLC (column; COSMOSIL 5C18-MS Waters 4.6 × 150 mm (Nacalai Tesque, Inc.), mobile phase; CH_3CN:0.1% AcOH aq (6: 4), flow rate; 1 mL/min, temperature; rt, absorbance; 235 nm).

Measurement of Fe^{3+}-ADP induced NADPH dependent lipid peroxidation in rat liver microsome.[22] The modified method of Kiso et al.[23] was used. Microsomes were prepared from male Wistar rats weighing about 200 g. The rats liver was homogenized in cold 0.25 M sucrose. The homogenate was centrifuged at 4 °C ($8000 \times g$, 10 min). The supernatant fraction was then collected and ultracentrifuged at 4 °C ($105{,}000 \times g$, 30 min). The pellet obtained was resuspended in 83.5 mM KCl–37.2 mM Tris–HCl buffer (pH 7.4) and stocked at −20 °C until use. Protein concentration was determined by the method of Lowry et al.[24] The assay system (1 mL) consisted of 83.5 mM KCl–37.2 mM Tris–HCl buffer (pH 7.4), the test compound in 1% DMSO, 0.2 mM NADPH, 1 mM ADP, 1 mg protein/mL rat liver microsomes and 10 μM $FeCl_3$. The reaction mixture was incubated at 37 °C for 20 min, and then cooled on ice to terminate the reaction. Lipid peroxide was measured by the method of Ohkawa et al.[25] Thus, 8.1% sodium dodecyl sulfate (0.2 mL), 20% AcOH containing 0.27 M HCl adjusted to pH 3.5 with NaOH (1.5 mL) and 0.8% TBA (1.5 mL) were added to the reaction mixture. The mixture was then boiled at 100 °C for 20 min and the reaction was stopped by cooling on ice. Thereafter, *n*-BuOH-pyridine (15:1, 4 mL) was added,

and vigorous mixing was performed. After centrifugation ($780 \times g$, 10 min), the organic layer was separated, and the absorbance was measured at 532 nm. The amount of TBA-positive material was expressed as a corresponding amount of malondialdehyde.

Effects on 48 h homologous PCA reaction in rats.[26] DNP-BSA and rat anti-DNP-BSA serum were used as antigen and antiserum, respectively. The dorsum of a male Wistar rat weighting about 200 g, was shaved and 0.1 mL of an antiserum, with a 48 h homologous PCA strength of 1:128–1:256 diluted with physiological saline, was injected into the dorsum intracutaneously. After 48 h, 1 mL of 0.5% Evans blue physiological saline, containing DNP-BSA equivalent to 1 mg protein, was injected into the caudal vein. After 30 min, the rat was killed by exsanguination and the pigment freckle generated on the back skin was cut out and the amount of transudate pigment was determined. To do this, the cut out skin was put in a test tube, 1 mL of 1 N KOH was added and allowed to stand overnight at 37 °C to elute the pigment and then 9 mL of a mixture of acetone and 0.6 N phosphoric acid (mixed at 13:5) was added with shaking. The insoluble substances were removed by centrifugation at $1500 \times g$ for 10 min, and the absorbence of the supernatant was measured at 620 nm to determine the amount of pigment. The specimen was suspended in 0.2% carboxymethyl cellulose sodium salt, and given orally to the rat, in dose of 0.5 mL per 100 g body weight, 1 h before antigen administration.

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